PURIFICATION AND PROPERTIES OF ARGININE PHOSPHOKINASE FROM HONEYBEES *APIS MELLIFERA* L. (HYMENOPTERA, APIDAE)

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Arginine phosphokinase was purified from honeybee thoraces. Its molecular weight was estimated by Sephadex gel chromatography at about 36,000. In the direction of arginine phosphate synthesis, the enzyme had a pH optimum around 8.3. The energy of activation for the reaction from 22-45 C was 7,000 cal/mole. Optimum molar ratio of Mg^{++}: ATP appeared to be 1:1. Besides Mg^{++}, the enzyme was activated to various extents by Mn^{++}, Ca^{++}, Co^{++}, and Cu^{++}. UTP, GTP, CTP, and ADP could not substitute for ATP as substrate. The enzyme phosphorylated L-arginine methyl ester and to a much less extent D-arginine, but did not phosphorylate creatine, guanidoacetic acid, nor hippuryl-L-arginine.

L'arginine phosphokinase a été purifiée à partir de thoraxes d'abeilles. Son poids moléculaire a été estimé par chromatographie sur gèle Sephadex à environ 36,000. Dans la réaction donnant la synthèse de l'arginine phosphate, l'enzime a un pH optimum aux environs de 8.3. L'énergie d'activation pour la réaction de 22-45 C était 7,000 cal./mole. Le rapport molaire optimum de Mg^{++}. ATP apparait être 1:1. En plus de Mg^{++}, l'enzime a été activée à différents degrés par Mn^{++}, Ca^{++}, Co^{++}, et Cu^{++}. UTP, GTP, CTP, et ADP ne peuvent pas remplacer l'ATP comme substrat. L'enzime phosphorilate L-arginine methyl ester et à un degré moins élevé, la D-arginine, mais ne phosphorilate pas la créatine, l'acide guanido-acetique, et la hippuryl-L-arginine.

Using crab-muscle extracts, Lohmann (1935) first discovered the enzyme arginine phosphokinase (EC 2.7.3.3) which catalyzed the reaction:

$$ATP + arginine \rightleftharpoons ADP + arginine\ phosphate + H^+$$

Since then other workers have described the enzyme from various invertebrates. Arginine phosphokinase has been purified or identified from extracts of the following species: freshwater crayfish *Potamobius astacus*, and *P. leptodactylus* (Elodi and Szorényi, 1956), sea crayfish *Jasus verreauxi* (Morrison *et al.*, 1957; Uhr *et al.*, 1966), shrimps *Palaemon serratus, P. elegans* (Virden and Watts, 1964), crabs *Pagurus bernhardus, Callinectus sapidus* (Blethan and Daplan, 1968), *Atelecyclus septemdentatus, Cancer pagurus, Portunas depurator, Carcinus maemas, Maia squinado* (Virden and Watts, 1964), lobsters *Homarus vulgaris* (Pradel *et al.*, 1964; Virden *et al.*, 1965), *H. americanus* (Blethan and Kaplan, 1967; Regnouf *et al.*, 1969), and *Nephrops norwegicus* (Virden and Watts, 1964), molluscs *Pecten maximus*, and *Chlamys opercularis* (Virden and Watts, 1964), the horseshoe crab *Limulus polyphemus* (Blethan and Kaplan, 1968), echinoderms *Centrostephanus rodgersii, Heliocidaris erythrogramma* (Griffiths *et al.*, 1957a), and *Echinus esculentus, Holothuria forskali,* and *Asterias rubens* (Virden and Watts, 1964), the tunicate *Styella mammiculata* (Virden and Watts, 1964), the cephalochordate *Amphioxus lanceolatus* (Virden and Watts, 1964), arachnids *Pholcus phalangioides*, and *Dugesiella hentzi* (Blethan and Kaplan, 1968), the annelid *Sipunculus nudus* (Regnouf *et al.*, 1969), insects *Melanoplus bruneri, Apis mellifera, Porthetria dispar, Sympetrum rubicundulum*, (Blethan and Kaplan, 1968), and *Calliphora erythrocephala* (Lewis and Fowler, 1962), protozoans *Tetrahymena pyriformis* (Robin and Viala, 1966; Watts and Bannister, 1970), and *Stentor coeruleus* (Watts *et al.*, 1968), and from the bacteria *Escherichia coli* (Di Jeso, 1967).

In the study of the properties of this enzyme most workers used enzymes extracted from

crustaceans. Little work has been done on the enzyme from insect sources. Recently Carlson *et al.* (1971) reported the crystallization of arginine kinase from honeybee thoraces. These workers indicated that the physical, chemical, and catalytic properties of the enzyme were being studied. So far there has been no report on the properties of an insect APK. I report a method of purifying arginine phosphokinase from honeybee thoraces and the investigations on some properties of the enzyme in the direction of arginine phosphate synthesis.

The following abbreviations are used: arginine phosphokinase, APK; arginine phosphate, AP; adenosine triphosphate, ATP; uridine triphosphate, UTP; guanosine triphosphate, GTP; cytidine triphosphate, CTP; adenosine diphosphate, ADP; diethyl aminoethyl cellulose, DEAE-cellulose; disodium ethylenediamine-tetraacetate, EDTA; and Tris (Hydroxymethyl) aminomethane, Tris.

MATERIALS AND METHODS

Experimental animals

Honeybee workers (*Apis mellifera* L.) were obtained from a local apiary and frozen until use.

Sources of chemicals

ATP and L-arginine came from both Sigma Chemical and Calbiochem. UTP, CTP, GTP, ADP, L-arginine methyl ester, hippuryl-L-arginine, guanidoacetic acid, creatine, cytochrome c, Tris, and DEAE-cellulose came from Sigma Chemical. D-arginine, myoglobin, haemoglobin, and bovine albumin came from Nutritional Biochemicals. L-cysteine HCl, 2-mercaptoethanol, reduced glutathion, γ-globulin, and Aquacide I came from Calbiochem. $MgSO_4$, $MnSO_4$, $CuSO_4$, $CoCl_2$, $CaCl_2$, ammonium molybdate, 1-amino-2-naphthol-4-sulfonic acid, and EDTA came from Fisher Scientific.

Assay procedure

The activity of the enzyme was estimated by measuring the inorganic phosphate released after acid hydrolysis of arginine phosphate. The assay procedure was modified from that of Morrison *et al.* (1957). The reaction mixture contained a final concentration of 50 mM Tris, 1 mM 2-mercaptoethanol, 5 mM ATP, and 10 mM each of arginine and $MgSO_4$, pH 8.3. In a typical assay, 0.9 ml of the stock solution was incubated at 30 C for 5 min, the reaction was started by adding 0.1 ml APK solution. The reaction was stopped after 5 min by adding 0.5 ml 30% acetic acid. The solution was placed in boiling water for exactly 1 min, after which it was immersed in an ice-bath. The colorimetric determination of inorganic phosphate was started by adding 2.0 ml 5% w/v ammonium molybdate in 15% v/v H_2SO_4 followed by 0.5 ml 0.25% aminonapthol sulfonic acid half a minute later. The mixture was diluted with 5 ml glass distilled water. The absorbance of the resulting blue solution was read at 540 nm in a Beckman DU-2 spectrophotometer after 20 min. Controls were run in the same way except that acetic acid was added before adding the enzyme. Enzyme activity was measured as the difference in absorbance between the 5 min assay and the control. When working with the crude extracts and various $(NH_4)_2SO_4$ fractions, correction for ATPase activity was made by subtracting the change in absorbance without arginine in the stock solution. By using a standard curve prepared with various amounts of inorganic phosphate, enzyme velocity was converted to μmoles arginine phosphate synthesized per min.

For higher temperatures or lower substrate concentrations, the duration of the assay was reduced to ensure that only the linear portion of the reaction velocity was measured.

Protein concentrations were determined spectrophotometrically according to the method of Layne (1957).

RESULTS

Purification of arginine phosphokinase

Purification procedure was carried out at 0-4 C. The buffer used was 10 mM Tris, 5 mM EDTA, pH 7.0, unless stated otherwise.

Extraction. – Bee thoraces weighing 50 g were homogenized in 150 ml Tris buffer with an omni-mixer for 3 min and centrifuged at 10,000 g for 30 min. The supernatant was saved. The above procedure was repeated on the precipitate with 100 ml buffer. The supernatants were combined.

Ammonium sulfate fractionation. – Granular $(NH_4)_2SO_4$ was added to the combined supernatant until 60% saturated. The precipitate formed after centrifugation at 10,000 g for 30 min was discarded. More $(NH_4)_2SO_4$ was added to the supernatant until 80% saturated and the precipitate collected after centrifugation at 10,000 g for 30 min was saved. Precipitate from this $(NH_4)_2SO_4$ fraction gave the highest specific activity reading. The reading was much lower than expected, probably due to the high concentration of SO_4 = ion which was a potent inhibitor of creatine phosphokinase (Noda *et al.*, 1960). Chloride, nitrate, and acetate as the sodium and potassium salts inhibited arginine phophokinase from lobster (Virden *et al.*, 1965).

Sephadex G-100 chromatography. – The precipitate from 80% $(NH_4)_2SO_4$ fraction was dissolved in Tris buffer and put through a Sephadex G-100 column (1.4 x 102 cm) equilibrated with 50 mM Tris, 5 mM EDTA, 100 mM KCl, pH 7.0 buffer. The column was eluted with the same buffer. Protein concentration of the eluent was estimated by measuring the absorbance at 280 nm and APK activity was assayed in the direction of AP synthesis as described under assay method.

DEAE-cellulose chromatography. – The solution from Sephadex chromatography was dialyzed against two changes of Tris buffer overnight. The dialyzed solution was pumped through a DEAE-cellulose column (2.5 x 24 cm) equilibrated with Tris buffer. The column was eluted by 0.1, 0.2, 0.4 M NaCl, and 30% $(NH_4)_2SO_4$ in Tris buffer. The enzyme appeared shortly after 0.1 M NaCl in Tris buffer was pumped into the column. The enzyme solution obtained was free of ATPase activity. A final concentration of 1 mM 2-mercaptoethanol was added to the enzyme solution. A summary of the data is listed in Table 1.

Table 1. Purification of arginine phosphokinase from honeybee thoraces. Weight of thoraces, 50 g.

Step	Vol. (ml)	Protein (mg)	Sp. Act. (μmoles/ min/mg protein)	Purification	Total Act. (μmoles/ min)	Yield (%)
Crude extract	222	910	0.29	1	264	100
$(NH_4)_2SO_4$ fractionation	11.4	340	0.08*	–	–	–
Sephadex G-100	47.5	95	1.83	6.3	174	66
DEAE-cellulose	125	5	33.7	116	167	63

* See text for the explanation of the exceptionally low specific activity.

Estimation of molecular weight by Sephadex G-100 gel chromatography

A Sephadex G-100 column (1.4 x 102 cm) was calibrated according to the method of Andrews (1964). The proteins used were γ-globulin, bovine albumin, haemoglobin, myoglobin, and cytochrome c. The molecular weight of the enzyme estimated by this method is 36,000 ± 3,000 (Fig. 1).

Electrophoresis of APK

The APK solution was concentrated by Aquacide I to a concentration of 9.6 mg/ml. About 5 μl was applied to each Sepraphore III cellulose polyacetate strip (1" x 6¾") and electrophoresed at a constant current of 1 ma/strip for 65 min. The buffer used was 10 mM Tris, 1 mM EDTA, 1 mM 2-mercaptoethanol, at pH's 9.0, 7.0, and 4.0. At these three pH's the enzyme migrated as a single protein band, APK activity coincided with the band. Protein was stained with Ponceau S.

Effect of pH on APK activity

The assays were done in 50 mM Tris, 100 mM bicarbonate buffer. Maximum activity occurred around pH 8.3. At pH's higher than 9.0, the enzyme activity declined rapidly (Fig. 2).

Effect of enzyme concentration on velocity

The reaction velocity was directly proportional to enzyme concentration over the concentration range studied (Fig. 3). The reaction time used was 5 min for APK concentrations up to 4 μg/ml; above this the reaction time was 2.5 min. This eliminated the chance of measuring the non-linear portion of the reaction velocity.

Temperature stability of the enzyme

Aliquots of the enzyme solution were incubated at various temperatures for 15 min and then assayed at 30 C for 15 min. There was no loss of activity up to 40 C, from 40-45 C activity declined slightly, and beyond 45 C a sharp decline in activity was observed (Fig. 4).

Effect of temperature on velocity

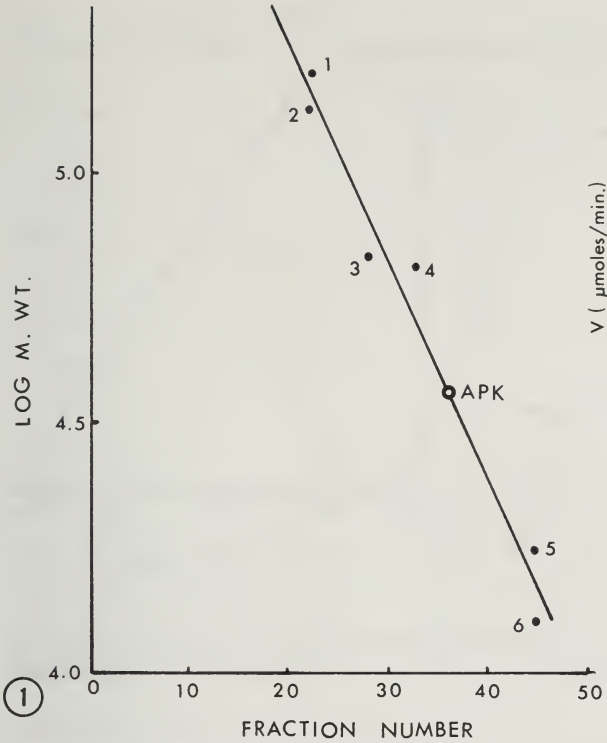
To ensure only the initial velocities were measured, the assays were shortened as the temperature was increased. The durations of the assays for 22, 29, 34, 39, 45, and 50 C were 5′, 5′, 3′, 2′, 1′, and 1′, respectively. Velocity increased from 22-45 C, beyond this temperature range velocity declined (Fig. 5). When the reciprocals of absolute temperatures for the range of 22-45 C were plotted against the logarithm of velocity, a linear Arrhenius plot was obtained (Fig. 6). The activation energy calculated from the slope of the graph was 7,000 cal/mole.

Effect of sulfhydryl compounds

Addition of sulfhydryl compounds to the assay solution increased the enzyme activity by as much as 33%. Cysteine, 2-mercaptoethanol, and reduced glutathion all had similar effects. The activation by 2-mercaptoethanol is shown in Fig. 7.

Activation by various bivalent cations

The metal salts used were either sulfates or chlorides. No activity was detected without adding bivalent cations. Addition of 10 mM of Mg^{++}, Mn^{++}, Cu^{++}, Ca^{++}, and Co^{++} activated the enzyme to various extents. The enzyme was more active with Mn^{++} than with Mg^{++}; Cu^{++}, Ca^{++}, Co^{++} activated the enzyme to a much less extent. These results are listed in Table 2.



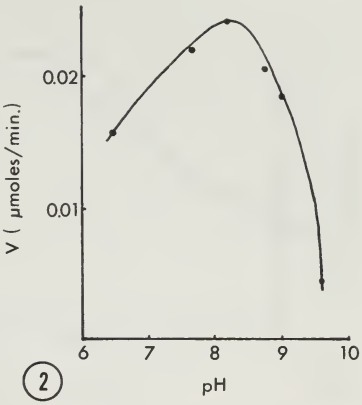
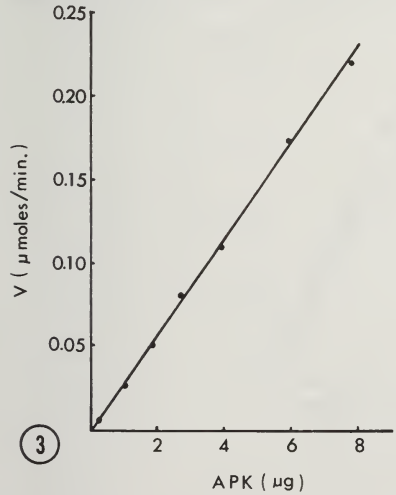


Fig. 1. Estimation of the molecular weight of honeybee APK by Sephadex G-100 gel chromatography. The proteins used and their molecular weights were: 1. γ-globulin, 160,000; 2. bovine albumin (dimer), 134,000; 3. bovine albumin (monomer), 67,000; 4. haemoglobin, 64,500; 5. myoglobin, 17,800; 6. cytochrome c, 12,400. The molecular weight of APK estimated by this method is 36,000 ± 3,000.

Fig. 2. Effect of pH on reaction velocity of honeybee APK. The buffer used was 50 mM Tris, 100 mM bicarbonate at various pH's. Optimum activity occurred around pH 8.3.



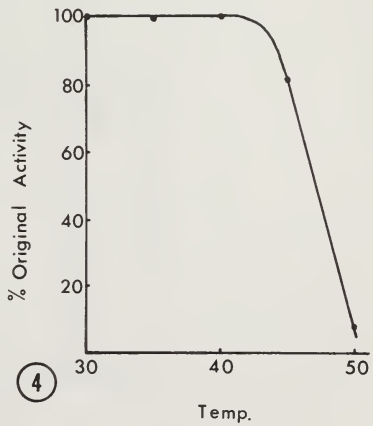


Fig. 3. Effect of enzyme concentration on the reaction velocity of honeybee APK. Reaction time was 5 min up to 4 μg APK added, above 4 μg, reaction time was 2.5 min.

Fig. 4. Temperature stability of honeybee APK. Aliquots of the enzyme were incubated at various temperatures for 15 min and then assayed at 30 C for 15 min.

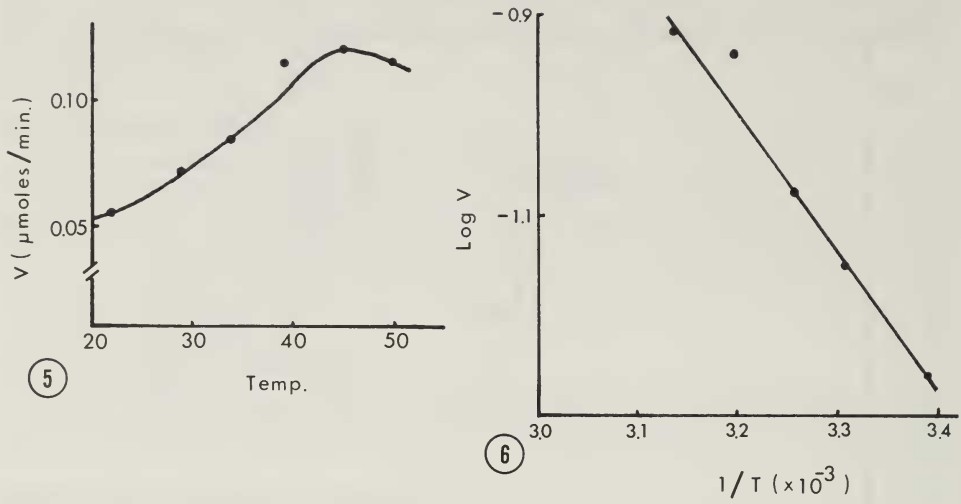


Fig. 5. Effect of temperature on reaction velocity of honeybee APK. Duration of the assays were progressively decreased as temperature was increased so that only the linear portion of the reaction velocity was measured. APK: 1.3 μg/assay.

Fig. 6. The Arrhenius plot of the data from Fig. 5. T = absolute temperature; v = μmoles/min. APK: 1.3 μg/assay.

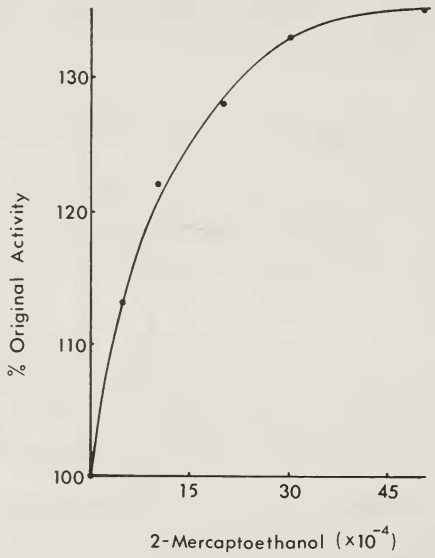


Fig. 7. Effect of 2-mercaptoethanol on reaction velocity of honeybee APK. With sulfhydryl-free APK and reaction solution, addition of 2-mercaptoethanol to the reaction solution increased the enzyme activity up to 33% of its original activity. APK: 1.3 μg/assay.

Table 2. Activation of APK by metal ions. Conditions as described in assay procedure except that 10 mM of the following ions was used in place of Mg^{++}. APK: 1.3 μg/assay.

Metal ion	Enzyme activity (μmoles/min)	Percentage activity
Mn^{++}	0.082	111
Mg^{++}	0.074	(100)
Ca^{++}	0.017	23
Co^{++}	0.010	13.5
Cu^{++}	0.006	8.1
None	0	0

Effect of varying magnesium concentration on velocity

The effect of increasing magnesium concentration on velocity was studied with three levels of ATP concentration (Fig. 8). The maximum velocity was reached when the molar ratio of Mg^{++} : ATP was one. Increase in the ratio caused a slight decline in velocity. Similar results were obtained when Mg^{++} was replaced by Mn^{++}. The Lineweaver-Burk plot of the reaction velocities against ATP concentrations at 5 mM and 10 mM Mg^{++} indicates that the inhibition by Mg^{++} was competitive.

Specificity of arginine phosphokinase

When ATP was replaced by the same concentration of UTP, CTP, GTP, or ADP, no activity was observed.

The ability of the enzyme to phosphorylate several guanidino compounds was determined. The final concentration of the guanidines in the reaction mixture was 10 mM. The results of the experiment are shown in Table 3. Both L-arginine methyl ester and D-arginine served as substrates to a limited extent.

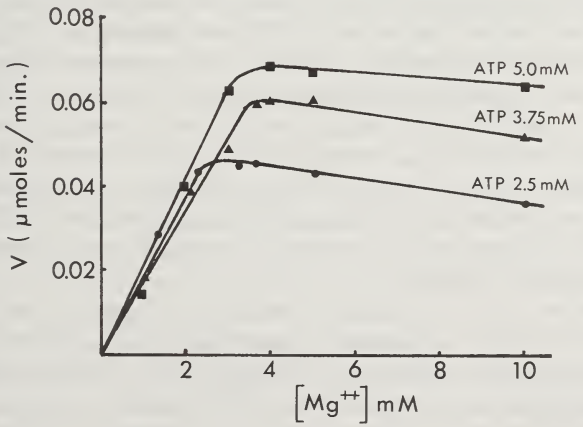


Fig. 8. Effect of magnesium concentration on reaction velocity of hoenybee APK at three ATP concentrations. Reaction time, 5 min; APK: 1.3 μg/assay.

Table 3. Specificity of APK: phosphorylation of guanidines. Conditions as described in assay procedure except that 10 mM of the following guanidines was used in place of L-arginine. APK: 2.6 μg/assay.

Guanidines	Enzyme activity (μmoles/min)	Percentage activity
L-arginine	0.13	(100)
L-arginine methyl ester	0.056	43
D-arginine	0.016	12.3
Guanidoacetic acid	0	0
Hippuryl-L-arginine	0	0
Creatine	0	0

DISCUSSION

Using sedimentation and diffusion experiments, Elodi and Szorényi (1956) estimated the molecular weight of APK from *Potamobius astacus* to be 43,000. Virden *et al.* (1966) concluded from estimates obtained with ultracentrifuge analysis, gel filtration, and density-gradient centrifugation, that the molecular weight of the enzyme from *Homarus vulgaris* was 37,000. Blethan and Kaplan (1968) estimated the molecular weights of APK from several arthropods by gel chromatography to range from 35,000 ± 2,000 to 38,000 ± 2,000. Moreland and Watts (1967) discovered the existence of two forms of APK in some molluscs; one with a molecular weight of 40,000 and the other 80,000. From the distribution of the isoenzymes in different muscle tissues, they suggested that the different forms of enzyme were associated with different muscle functions and structures. Regnouf *et al.* (1969) had shown that APK from *Homarus vulgaris* had a molecular weight of 43,000 and consisted of a single polypeptide chain, whereas APK from the annelid *Sipunculus nudus* with molecular weight of 86,000 was a dimer. Oriol *et al.* (1970) again showed that APK's from lobster and crab with molecular weights of about 40,000 were monomers. Robin *et al.* (1969) identified an APK from the polychaetes *Sabella pavonina* and *Spirographis spallanzanii* with a molecular weight of 160,000. Thus, various workers have shown that there are at least three forms of APK in invertebrates, a monomer with a molecular weight of about 40,000, a dimer, and a tetramer, with corresponding molecular weights. In the present study, both gel chromatography and electrophoresis indicated that only one form of APK was present in honeybee thoraces, and from gel chromatography, the molecular weight of the enzyme was estimated to be 36,000 ± 3,000.

The honeybee enzyme has a pH optimum around 8.3. The activity declines sharply at pH's higher than the optimum and becomes insignificant beyond pH 9.5. Virden *et al.* (1965) had similar results with APK from *Homarus vulgaris*. However, Morrison *et al.* (1957) reported a pH optimum of 8.4-8.5 with APK from *Jasus verreauxi* with a much broader pH tolerance. Whether this difference in pH tolerance is due to differences between the enzymes or to experimental conditions is still to be investigated.

As in *Homarus vulgaris* (Virden *et al.*, 1965), APK from honeybees is activated by Ca^{++} and Co^{++}. The enzyme from sea crayfish was not activated by either Ca^{++} or Co^{++} (Morrison *et al.*, 1957).

The enzyme is quite specific with respect to the nucleotide substrate. ATP cannot be substituted by UTP, CTP, GTP, or ADP. It is less specific with the guanidino substrate. The enzyme is able to phosphorylate L-arginine methyl ester and to a much less extent, D-arginine. APK from *Sabella pavonina* was reported to show significant activity with D-arginine (28% of the activity with L-arginine) and those from *Maia squinado, Eupagurus bernhardus, Pecten maximum, Polycelis cornuta, Myxicola infundibulum,* and *Holothuria forskali* were also reported to have some activity with D-arginine (1-7% of the activity with L-arginine) (Virden and Watts, 1964).

Without added metal ions, no activity was observed. A similar finding was reported by Virden *et al.* (1965) with lobster enzyme, and a trace of activity was reported by Morrison *et al.* (1957) with crayfish enzyme. Optimum molar ratio of Mg^{++} : ATP appeared to be 1:1, similar to the findings of Griffiths *et al.* (1957b). An increase in the ratio led to a slight decline in activity.

Preliminary studies of initial velocity and product inhibition indicated that the reaction mechanism is random sequential.

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